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Tumor mutation burden and immunological, genomic, and clinicopathological factors as biomarkers for checkpoint inhibitor treatment of patients with non-small-cell lung cancer

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Abstract

Cancer treatment using immune checkpoint inhibitors is widely used, although biomarkers predictive of response are not well-established. However, both the expression of programmed cell death ligand 1 (PD-L1) and the tumor mutation burden (TMB) hold promise as such biomarkers for immune checkpoint inhibitors; however, its characteristics and clinical and immunological impacts have not been fully analyzed. We therefore evaluated the clinical and immunological parameters related to TMB to identify potential new biomarkers. We enrolled 92 patients with non-small-cell lung cancer who underwent surgery at Fukushima Medical University Hospital from 2013 to 2016. TMB of individual tumors was calculated by whole-exome sequencing analysis. Major cancer-related gene mutations were evaluated using panel sequencing. Expression of PD-L1 and abundance of tumor-infiltrating lymphocytes were evaluated by immunohistochemistry using surgical samples. The median TMB value was 60. TMB was significantly higher in men, current or former smokers, and in patients with squamous cell carcinoma,

tumor size ≥ 2.8 cm, wild-type EGFR, TP53 gene mutation-positive status, and cyclin-dependent kinase-inhibitor gene 2A mutation-positive status. According to multivariate analysis, TMB was significantly associated with EGFR gene mutation-negative status ($p=0.0111$) and TP53 gene mutation-positive status ($p=0.0425$). If TMB is identified as a robust biomarker for immune checkpoint inhibitor administration, analysis of TP53 and EGFR mutations may provide a relatively rapid and easy proxy for predicting TMB.

Abbreviations

MSI	microsatellite instability
NGS	next-generation sequencing
NSCLC	non-small-cell lung cancer
PD-1	programmed cell death 1
PD-L1	programmed death ligand 1
TIL	tumor-infiltrating lymphocyte
TMB	tumor mutation burden
TP53	tumor protein 53

Introduction

Developments in immune checkpoint inhibitors have progressed rapidly, and they are now major pillars of cancer treatment, along with cytotoxic anticancer drugs and molecular-targeted therapeutic agents. Several immune checkpoint inhibitors, including programmed cell death 1 (PD-1) inhibitors and programmed-death ligand 1 (PD-L1) inhibitors, were recently approved by the US Food and Drug Administration for the treatment of advanced non-small-cell lung cancer (NSCLC). PD-L1 is the primary PD-1 ligand and is upregulated in many solid tumors. PD-L1 is believed to inhibit cytokine production and the cytolytic activity of PD-1-positive tumor-infiltrating lymphocytes (TILs). Most prospective trials found that the treatment was more effective in PD-L1-positive compared with PD-L1-negative patients. However, the PD-L1-negative group still demonstrated response rates of around 10%, indicating that PD-L1 was not a perfect predictive biomarker for response (1-8). More definite biomarkers are therefore needed, and many studies are currently addressing this issue.

Several parameters other than PD-L1 expression (TILs, microsatellite instability (MSI), and tumor mutation burden (TMB)) have been considered as potential predictive biomarkers of immune checkpoint inhibitor response. Pre-existing CD8⁺TILs located in the tumor and invasive margin might predict response to therapy in patients with melanoma (9). Although MSI is often used as a biomarker in colorectal cancer and Lynch syndrome, high MSI was only detected in 0.8% of 480 patients with pulmonary adenocarcinoma using a sensitive mononucleotide marker panel (10). MSI is currently used as a companion diagnostic technique in various tumors; however the rate of MSI in NSCLC is thought to be very low, indicating the need for more accurate and clinically useful biomarkers. Several

solid tumors, including NSCLC, have TMB levels >10 somatic mutations per megabase of coding DNA, which is sufficient to produce neoantigens that can be recognized by effector-T cells (11). Among these potential parameters (PD-L1, CD8+TILs, MSI, and TMB), the current study focused on the use of TMB to predict the efficacy of immune checkpoint inhibitors. A higher nonsynonymous TMB was correlated with the clinical efficacy of pembrolizumab in one study (12), and although several other reports have been published, detailed information on TMB remains limited. In a biomarker analysis of TMB conducted as a subgroup analysis of the Check-mate 026 trial, which compared first-line nivolumab with chemotherapy in patients with PD-L1-positive NSCLC, the response rate among patients with high TMB was higher in the nivolumab group than in the chemotherapy group (13). Furthermore, patients with both high TMB and $\geq 50\%$ PD-L1 expression had higher response rates than those with only one or neither of these factors. Most TMB studies have evaluated the association between TMB and response to immune checkpoint inhibitors, while the associated clinical features have not been well-documented. In this study, we therefore evaluated the correlations between TMB and clinical and immunological parameters in patients with NSCLC, with the aim of identifying more convenient factors to use as surrogate markers for TMB.

Materials and Methods

Patients and characteristics

We enrolled a total of 92 patients who underwent surgery at the Hospital of Fukushima Medical University from 2013–2016. No patients received chemotherapy or immunotherapy before surgery. Disease staging was evaluated

according to the current International Union Against Cancer TNM classification, 7th edition. Paired tumor and normal tissues dissected from surgical specimens were collected from all 92 patients for whole-exome sequencing and immunohistochemistry.

Whole-exome sequencing

The 92 pairs of matched tumor and non-tumor samples (184 samples in total) were subjected to whole-exome next-generation sequencing (NGS) using an Ion AmpliSeq™ Exome technology and Ion Proton™ platform (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Briefly, exome libraries were prepared using an Ion AmpliSeq Exome RDY Kit (Thermo Fisher Scientific) with 100 ng of genomic DNA extracted from the paired tumor and adjacent non-tumor tissue (or corresponding peripheral blood sample) for target amplification by PCR, as described in the manufacturer's protocol. The obtained libraries were optimized using an Ion Library Equalizer kit (Thermo Fisher Scientific) and then sequenced using an Ion Proton or Ion S5XL platform (Thermo Fisher Scientific). The sequenced reads were aligned to the reference genome build hg19 and GRCh37, and converted into binary alignment map files using Ion Torrent Suite software (Thermo Fisher Scientific). The average of Q20 bases and mean coverage depth of the 184 samples were 6.44 Gbp and 123×, respectively, and 90.4% of target bases had a coverage of 20×. Sequence variants found only in tumors were called using Ion Reporter™ 5.0 (Thermo Fisher Scientific) and CLC Genomics Workbench 8.0 software (Qiagen, Hilden, Germany), and the number of nonsynonymous coding variants was counted. The resulting value was designated as the TMB.

Tumor variants within the hotspot regions for the following genes were detected using the Ion Ampliseq™ Colon and Lung Cancer Panel v2 (Thermo Fisher Scientific) and Ion Personal Genome Machine™ (PGM™ platform (Thermo Fisher Scientific): EGFR, TP53, KRAS, ERBB2, BRAF, CTNNB1, PTEN, cyclin-dependent kinase-inhibitor 2A (CDKN2A), and PIK3CA. Briefly, 10 ng of genomic DNA extracted from the 92 pairs of matched tumor and non-tumor samples was used to prepare a DNA library, as described in the manufacturer's instructions. Mutation hotspots in CDKN2A were also sequenced using the Ion Ampliseq™ Cancer Hotspot Panel v2 (Thermo Fisher Scientific) using Ion PGM™, according to the manufacturer's instructions.

Immunohistochemistry

Fresh-frozen paraffin-embedded tissue sections of 4-μm thickness were stained for PD-L1 as described previously (14). Sections were also stained for CD8 to evaluate CD8⁺TILs, and p53 to evaluate p53 protein expression. The sections were dewaxed in xylene and dehydrated through an alcohol gradient. Endogenous peroxidase activity was quenched by 20-min incubation with a 0.3% (v/v) solution of hydrogen peroxidase (Wako Pure Chemical Industries Ltd., Osaka, Japan) in 100% methanol. The sections were then incubated in 5% dried skimmed milk in phosphate-buffered saline for 30 min at room temperature, and incubated overnight at 4°C with primary monoclonal antibodies to PD-L1 (1:100; clone SP142; Ventana, Tucson, AZ, USA), CD8 (1:50; clone C8/144B; DAKO, Santa Clara, CA, USA), or p53 (1:500; Bp53-12, anti-human p53 protein monoclonal antibody, Santa Cruz Biotechnology, TX, USA) using the avidin-biotin complex method. The sections were washed several times in phosphate-buffered saline after

each step and counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Co., Ltd., Tokyo, Japan), dehydrated through an alcohol gradient, and mounted on glass slides.

PD-L1-positivity was determined as >1% tumor area infiltrated by PD-L1-positive immune cells (tumor cell: TC1 or immune cell: IC1) for the SP142 (15). CD8+TILs were classified as low (<30%), intermediate (30%–60%), or high (>60%) according to the positive rate of CD8 staining (16). p53-positivity was defined by nuclear staining as 0 (absence of p53- positive cells), 1 (low p53, <5%), 2 (intermediate p53, 5%–50%), and 3 (high p53, >5%) (17).

Statistical analyses

The associations between TMB and clinical/immunological parameters were evaluated by univariate analysis using the Mann–Whitney test. Multivariate analysis was performed by multiple linear regression analysis. We estimated the correlation between TP53 mutation and p53 protein expression using Pearson's correlation coefficient. Multivariate analysis was conducted using SPSS version 23 (IBM, Armonk, NY, USA) and Graph Pad Prism version 7 (GraphPad Software, CA, USA) was used for all other statistical analyses.

Results

Patients and characteristics

A total of 92 patients were enrolled and their characteristics are summarized in Supplementary Table 1. The median age was 70 years, 70.7% of patients were male, and 69.6% of all patients were current or former smokers (median

Brinkman Index 700, range 45–2580). Sixty-four patients (69.6%) were diagnosed with adenocarcinoma based on surgical specimens. In terms of pathological staging, 73.9% of all patients were stage I. Lung cancer recurrence was seen in 22 patients (24.2%) except one patient with stage IV adenocarcinoma diagnosed at surgery. Overall 14.1% of patients died.

TMB analysis using NGS

We evaluated the TMB in surgical samples from the 92 patients by NGS. The median TMB was 60 somatic mutations per megabase of coding DNA (range 10–502) (Fig. 1). The most common type of mutation was missense mutations. The clinical/immunological parameters and mutations in cancer-associated genes obtained by panel sequencing are shown in Fig. 1, and correlations between these parameters and TMB obtained by univariate analysis are shown in Table 1. Regarding the clinical parameters, male sex ($p<0.0001$), current or former smoking status ($p<0.0001$), squamous cell carcinoma ($p<0.0001$), and tumor size ≥ 2.8 cm ($p=0.0218$) were significantly correlated with higher TMB. In terms of cancer-associated mutations, wild-type EGFR ($p<0.0001$), TP53 mutation positive ($p<0.0001$), and CDKN2A mutation positive ($p=0.0196$) were significantly related to TMB. EGFR mutation-negative and TP53 mutation-positive status significantly contributed to TMB based on multivariate analysis ($p=0.0111$ and $p=0.0425$, respectively) (Supplementary Table 2). We derived the following equation to predict TMB level:
$$\text{TMB} = 55.461 - 10.009 \times (\text{male: } 1, \text{ female: } 0) + 0.028 \times \text{Brinkman Index} + 18.380 \times (\text{squamous cell carcinoma: } 1, \text{ adenocarcinoma: } 0) + 7.581 \times \text{tumor size (cm)} - 65.327 \times (\text{EGFR+: } 1, \text{ EGFR-: } 0) + 47.050 - (\text{TP53+: } 1, \text{ TP53-: } 0)$$

1, TP53-: 0). The coefficient of determination (R^2) was 0.260, indicating a weak correlation.

Among the 65 EGFR-mutation-negative patients (Supplementary Table 3), male sex ($p=0.0296$), current or former smoker ($p=0.0022$), squamous cell carcinoma ($p=0.0034$), and TP53 alteration ($p=0.0006$) were correlated with TMB according to univariate analysis, and a significant association between TMB and TP53 was identified by multivariate analysis (Supplementary Tables 4 and 5). There was no association between TMB and CD8+TILs ($p=0.2973$) or TMB and PD-L1 ($p=0.1984$) (Supplementary Fig. 1).

Immunohistochemistry

Immunohistochemistry revealed that 12.7% of the tumors expressed PD-L1, but there was no correlation between PD-L1 and TMB. High CD8+TILs were detected in 23 (25.0%), intermediate in 53 (57.6%), and low in 16 (17.4%), with no correlation between CD8+TILs and TMB. There was also no significant correlation between PD-L1 and CD8+TILs. p53 protein expression in tumors was strongly related to TP53 mutation status measured by immunochemical staining and whole-exome sequencing, respectively ($r=0.6599$, $p<0.0001$) (Supplementary Fig. 2).

Discussion

The results of the current study indicated that higher TMB was strongly associated with both TP53 mutation-positive and EGFR mutation-negative status, while TMB was also significantly correlated with TP53 mutation-

positive status among EGFR mutation-negative patients. Notably, we found a lack of correlation between TMB and CD8+TILs, and between TMB and PD-L1 expression. All these parameters have been reported to play essential roles in immuno-oncology (18-20), and might also be predictive biomarkers for the efficacy of immune checkpoint inhibitors. The current results suggest that TMB, CD8+TILs, and PD-L1 are independent factors.

PD-L1 is currently the only clinical biomarker predicting a reliable effect of the anti-PD-1 antibody pembrolizumab for first-line therapy; however, even PD-L1-negative patients showed better survival outcomes with pembrolizumab compared with chemotherapy (12.6 versus 8.9 months, respectively) (21). Anti-PD-L1 inhibitors are also effective in approximately 10% of PD-L1-negative patients. To explain why PD-L1-negative patients benefit from immune checkpoint inhibitors, it is necessary to evaluate complex immunological microenvironments (20) and comprehensively consider the clinical events related to TMB and TILs.

The current study found that TMB was not correlated with either PD-L1 or CD8+TILs. Several previous studies also found no association between TMB and PD-L1 expression (13, 18). Although several previous studies have analyzed the relationship between TMB and TILs (22, 23), to the best of our knowledge, none have reported on the relationship between TMB and CD8+TILs. We also showed that PD-L1 expression in tumors was not correlated with the amount of CD8+TILs, indicating that TMB, PD-L1, and CD8+TILs may independently influence the effect of immune checkpoint inhibitors. However, immune-cell PD-L1 expression and infiltration into tumors might correlate with nonsynonymous mutations and tumor number in patients with large-cell neuroendocrine carcinoma (24). Multiple factors must thus be considered in relation to biomarkers of immune checkpoint inhibitors. Blank et

al. and Karasaki et al. previously suggested the concept of ‘cancer immunograms’ representing several immunological factors as a spider plot, which might be helpful for guiding personalized immunotherapy (25, 26).

Human cancers with higher TMB have been considered suitable for immunotherapy, because a higher TMB may be associated with more neoantigens (11, 27). Patients with higher TMB levels are more likely to benefit from immunotherapy using immune checkpoint inhibitors (12, 14, 28). It should be noted that the TMB in the current study was lower than in previous studies. This may be because we only counted nonsynonymous variants in tumor tissues compared with normal lung tissue, and there were therefore fewer nonsynonymous variants because adjacent lung tissue, rather than peripheral blood, was used for germline comparison. A high TMB can enrich neoantigen-specific T cells, which attack tumors and subsequently lead to successful treatment outcomes (11). However, there is currently no evidence to support the ability of TMB alone to predict the efficacy of immune checkpoint inhibitors, and although both high TMB and high PD-L1 expression are known to predict the effectiveness of anti-PD-1/PD-L1 inhibitors (13, 18), neither marker alone is sufficiently accurate. Both TMB and PD-L1 are tumor characteristics, thus highlighting the importance of patient immunological status. It might thus be necessary to analyze both tumor-specific parameters and the general conditions of patients to predict the benefits of immune checkpoint inhibitors.

Neoantigen-specific T cells mobilized by various gene mutations play a major role in tumor immunity, indicating the importance of the presence of TILs and T-cell activation. Regarding the classification of the tumor microenvironment, Teng et al. proposed four categories based on PD-L1 status and the amount of TILs (16). Furthermore, although the correlation between PD-L1 expression and TILs has been investigated in NSCLC, the

results were controversial (24, 29, 30). Immune-cell infiltration appears to be related to nonsynonymous mutations in the tumor (30), but studies showing a correlation between TMB and TILs are lacking. Unlike melanoma, it is difficult to investigate TILs in patients with advanced or recurrent NSCLC because of difficulties in obtaining sufficient tissue samples. Although we analyzed TILs in surgical specimens in the current study, it was difficult to predict how many lymphocytes infiltrated the tumor before administering immune checkpoint inhibitors based on smaller sample volumes such as bronchoscopic biopsies.

The following results were also derived from the current whole-exome sequencing. Genes related to the mismatch repair system were examined to identify candidate genes determining TMB. However, we detected no somatically-altered variants in the MLH1, MSH2, MSH6, and PMS2 genes in any of the 92 cases. Regarding other mismatch repair system gene groups, somatically altered variants were only detected in one or two cases per gene (data not shown). The rare detection of MSI in lung cancer was similar to previous reports (10, 31). In addition, among the genes with many detected variants, such as those for squamous cell carcinoma, it was difficult to relate these results to the TMB (data not shown).

Whole-exome sequencing is becoming widely used in major research institutions, and genetic analysis is thus becoming more common (32). Targeted panel sequences focusing on cancer-related genes are also now available. Targeted panel sequencing analysis could provide a surrogate marker for TMB (33, 34), and may be easier to introduce for clinical use. Furthermore, the cost of using whole-exome sequencing to determine TMB is about five- to ten-fold that of using Cancer Hotspot Panel sequencing to determine TP53 and EGFR. However, it may be

difficult to introduce panel sequencing for large numbers of patients worldwide (35). Predicting TMB by analyzing specific gene alterations such as TP53 may represent a useful alternative approach. TP53 is a well-known major regulator and repairer of genomic damage, and may thus also affect the TMB. However, our results suggested that EGFR mutation, unlike TP53 mutation, was not associated with a high mutation load. Fast growth and division do not necessarily produce many genetic mutations. Driver mutations, such as EGFR gene mutations, are known to be a strong oncogenic phenomenon, while situations without driver mutations may require more gene alterations to be oncogenic. The rates of cancer cell proliferation and division do not seem to depend on the presence of driver mutations or the diversity of other gene mutations. However, further studies are needed to clarify this essential oncologic issue.

The p53 protein is encoded by the TP53 gene, and TP53 gene mutation increases the expression of p53. Overexpression of p53 protein in tumors without lymph node metastasis is an independent adverse prognostic factor in patients with NSCLC, with 5-year survival rates of 74.1% and 37.5% in p53-negative and p53-positive node-negative patients, respectively ($p=0.022$) (36). p53 protein expression is thought to increase in line with cancer growth and progression (17). Both TP53 mutations and TMB tend to increase with tumor growth, as supported by the current correlation between TP53 mutation and TMB. Although this correlation was revealed by univariate analysis in our study, TMB was significantly higher in larger tumors (diameter ≥ 2.8 cm), indicating that TMB and TP53 reflected tumor growth. We have used the term ‘growth’ rather than ‘progression’, because there is no correlation between stage and TMB, and TMB and TP53 are thought to be affected by local tumor growth rather

than progression.

In conclusion, TMB may be associated with aberrations in the tumor suppressor gene TP53. Given that TMB is considered as a powerful potential biomarker for immune checkpoint inhibitors, it is possible that TP53 may contribute to predicting the benefit of immune checkpoint inhibitors. However, the current study did not demonstrate an association between TP53 and clinical outcome in patients using immune checkpoint inhibitors. Nevertheless, if TMB is recognized as a robust biomarker of response to immune checkpoint inhibitors, it is possible that analyzing TP53 and EGFR mutations may provide a rapid and easy proxy for predicting TMB. Although TMB was poorly correlated with TILs and PD-L1, future biomarkers involving combinations of several factors are likely to become more important in the future. Further studies are necessary to confirm our results and to assess the value of TP53 as a predictive biomarker of response to immune checkpoint inhibitors in patients with NSCLC.

Author contributions

Yuki Ozaki and Hiroyuki Suzuki designed the study. Yuki Ozaki wrote the initial draft of the manuscript. Satoshi Muto, Daisuke Tanaka, Hideaki Nanamiya, Jun-ichi Imai, Takao Isogai, and Shinya Watanabe contributed to analysis and interpretation of data, and assisted in the preparation of the manuscript. Satoshi Muto, Hironori Takagi, Masayuki Watanabe, Takuya Inoue, Mitsuro Fukuhara, Takumi Yamaura, Naoyuki Okabe, Yuki Matsumura, Takeo Hasegawa, Jun Ohsugi, Mika Hoshino, and Yutaka Shio contributed to data collection and interpretation, and

critically reviewed the manuscript. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Compliance with ethical standards

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval and ethical standards

This study was approved by the Institutional Ethics Committee at Fukushima Medical University (No. 2538).

Whole-exome sequencing by next-generation sequencing was performed in accordance with the Ethical Guidelines for Human Genome and Genetic Analysis Research.

Informed consent

Patients with lung cancer provided written informed consent for the use (including the use for NGS) of tissue specimens and clinical data for research prior to undergoing pulmonary resection at the Department of Chest Surgery of Fukushima Medical University.

Figure legends

Fig. 1 TMB and clinical/immunological parameters. Bar chart showing TMB for each patient, with bars in descending order of TMB. The most common mutation type was missense mutations. The patients' clinical features are described immediately underneath the bar, and gene variants according to panel sequence analysis are described in the bottom panel. Colored cells on the left indicate the state or positive mutation, and tumor diameter ≥ 2.8 cm

TMB: tumor mutation burden, Sq: squamous cell carcinoma, LN: lymph node, TIL: tumor-infiltrating lymphocyte, PD-L1: programmed cell-death ligand 1, IHC: immunohistochemical staining, TP53; tumor protein 53, KRAS; v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, ERBB2; human epidermal growth factor receptor 2, BRAF; v-raf murine sarcoma viral oncogene homolog B1, CTNNB1; catenin beta-1, PTEN; phosphatase and tensin homolog deleted from chromosome 10, CDKN2A: cyclin-dependent kinase-inhibitor gene 2A, PIK3CA; phosphoinositide-3-kinase, catalytic alpha polypeptide

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Table 1. Univariate analysis for predicting TMB.

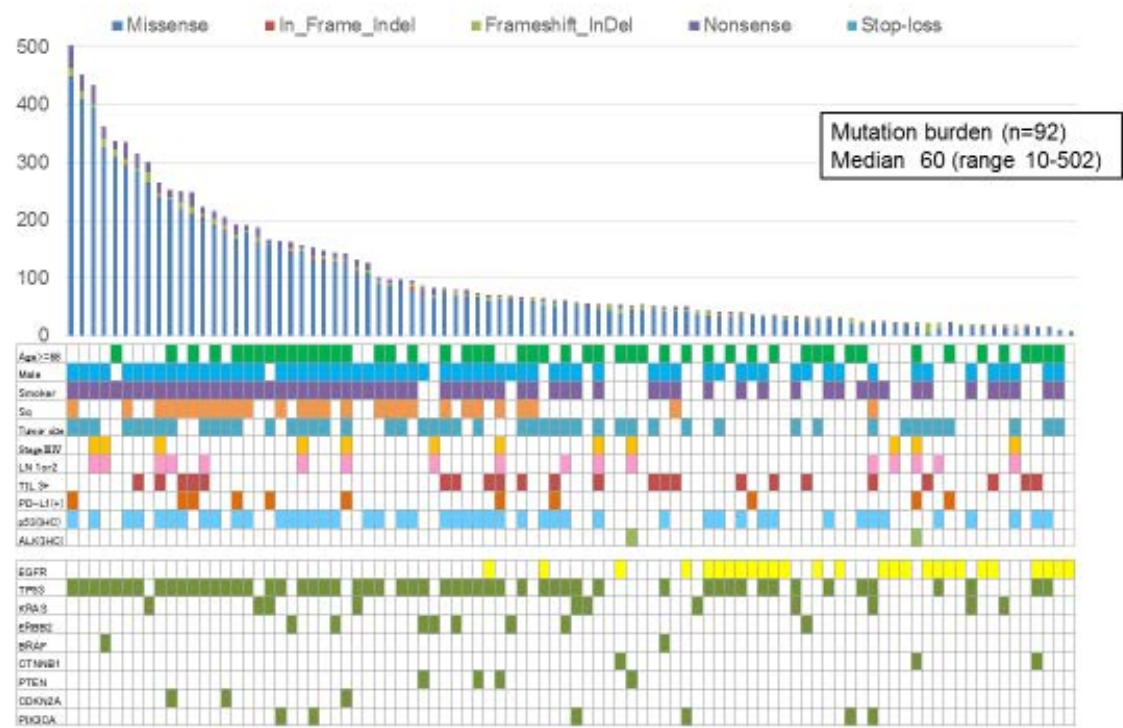
		n	mean	95%CI	p value
EGFR	Positive	27	32.0	25.8–38.2	<0.0001
	Negative	65	136.0	107.3–164.8	
TP53	Positive	56	138.0	105.7–170.3	<0.0001
	Negative	36	55.0	35.1–74.9	
KRAS	Positive	11	94.8	33.6–156.0	0.7871
	Negative	81	107.0	82.4–131.6	
ERBB2	Positive	8	81.5	53.7–125.6	0.3653
	Negative	84	54.5	82.5–131.5	
BRAF	Positive	2	207.0	0.0–2189.2	0.3987
	Negative	90	60.0	81.0–125.5	
CTNNB1	Positive	3	30.7	0.0–81.8	0.0893
	Negative	89	108.0	85.0–131.1	
PTEN	Positive	4	70.8	49.0–92.5	0.6743
	Negative	88	107.1	83.6–130.6	
CDKN2A ^a	Positive	3	199.7	62.6–336.8	0.0196
	Negative	55	80.7	57.5–103.8	
PIK3CA	Positive	6	80.0	15.2–144.8	0.8321
	Negative	86	107.3	83.5–131.1	
Age	>68	50	91.0	69.7–112.3	0.9363
	<67	42	122.8	79.9–165.7	
Sex	Male	65	127.5	98.8–156.2	<0.0001
	Female	27	52.5	27.0–78.1	
Smoking	Smoker	64	134.0	104.3–163.8	<0.0001
	Never	28	40.4	32.5–48.2	
Histology	Ad	64	80.5	55.0–106.0	<0.0001
	Sq	28	162.6	122.7–202.5	
Tumor size (average2.7)	≥2.8	48	128.4	82.6–164.1	0.0218
	<2.7	44	80.6	54.8–106.5	
Stage	I II	80	100.2	77.3–123.1	0.3719
	III IV	12	140.8	51.3–123.2	
LN	+	17	133.8	66.9–200.6	0.3833
	–	75	99.1	75.4–122.9	

p53 IHC	1–3+	47	122.1	89.3–155.0	0.0249
	–	45	88.2	57.1–119.2	
ALK IHC	Positive	2	38.0	0.0–228.6	0.3586
	Negative	90	107.0	84.1–129.9	
CD8+TIL	3+	23	93.5	53.1–133.9	0.6688
	1+/2+	69	109.5	82.2–136.8	
PD–L1	+	11	156.3	60.2–252.3	0.1479
	–	81	98.6	76–121.3	

^aCDKN2A mutation was identified using a CHP panel only, while other mutations were identified using CLP panel sequences. Therefore, the total number of CDKN2A analyses was 58, which differed from other CLP panel sequence analyses.

TMB: tumor-mutation burden, TP53; tumor protein 53, KRAS; v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, ERBB2; human epidermal growth factor receptor 2, BRAF; v-raf murine sarcoma viral oncogene homolog B1, CTNNB1; catenin, beta-1, PTEN; phosphatase and tensin homolog deleted from chromosome 10, CDKN2A: cyclin-dependent kinase-inhibitor gene 2A, PIK3CA; phosphoinositide-3-kinase, catalytic, alpha polypeptide, CHP: cancer hotspot panel, CLP: colon and lung cancer research panel, Ad: adenocarcinoma, Sq: squamous cell carcinoma, LN: lymph node, IHC: immunohistochemical staining, ALK; anaplastic lymphoma kinase, CD8; cluster of differentiation 8, TIL; tumor infiltrating lymphocyte, PD-L1; programmed cell death ligand 1

Figure 1



Supplementary Tables

Table S1. Patients' characteristics.

N=92			
Median age (range)		70 (40–87)	
Gender	Male / female	65 (70.7%)	/ 27 (29.3%)
Smoking status	Never smoker	28	(30.4%)
	Past or current smoker	64	(69.6%)
Median brinkman Index (range)		700 (45–2580)	
Tumor size (cm)		2.8 (0.8–11.0)	
Histology	Adenocarcinoma	64	(69.6%)
	Squamous cell carcinoma	28	(30.4%)
PD-L1	+ / –	11 (12.0%)	/ 81 (88.0%)
TIL	high / intermediate / low	23 (25.0%)	/ 53 (57.6%) / 16 (17.3%)
Pathological stage	I A	44	(47.8%)
	I B	24	(26.1%)
	II A	4	(4.3%)
	II B	8	(8.7%)
	III A	9	(9.8%)
	III B	2	(2.2%)
	IV	1	(1.1%)
EGFR-mutation	Exon21;L858R	17	(18.5%)
	Exon19 deletion	8	(8.7%)
	Others	2	(2.2%)
	Wild type/Unknown	65	(70.6%)

PD-L1: programmed cell-death ligand 1, TIL: tumor-infiltrating lymphocytes

Table S2. Multivariate analysis for predicting TMB.

		β	95%CI	p value
Gender	Male	-10.009	-38.90–18.88	0.7298
Brinkman Index		0.028	0.01–0.05	0.1885
Pathology	Sq	18.380	-7.30–44.07	0.4763
Tumor size		7.581	1.72–13.44	0.1994
EGFR	Positive	-65.327	-90.50–(-40.16)	0.0111
TP53	Positive	47.050	24.20–69.90	0.0425

CorrectedR²=0.260

TMB, tumor-mutation burden, Sq: squamous cell carcinoma

Table S3. Characteristics of patients without the EGFR mutation.

N=65			
Median age (range)		70 (40–87)	
Gender	Male / female	54 (83.1%)	11 (16.9%)
Smoking status	Past or current smoker	54	(83.1%)
	Never smoker	11	(16.9%)
Median brinkman Index (range)		840 (120–2580)	
Tumor size (cm)		2.9 (0.8–11.0)	
Histology	Adenocarcinoma	37	(56.9%)
	Squamous cell carcinoma	28	(43.1%)
PD-L1	+ / -	9 (13.8%)	56 (86.2%)
TIL	high / intermediate / low	17 (26.1%)	37 (56.9%) / 11 (16.9%)
Pathological stage	I A	29	(44.6%)
	I B	16	(24.6%)
	II A	4	(6.2%)
	II B	5	(7.7%)
	III A	9	(13.8%)
	III B	1	(1.5%)
	IV	1	(1.5%)

EGFR: EGFR gene

Table S4. Univariate analysis for predicting TMB in EGFR-mutation-negative patients.

		n	mean	95%CI	p value
TP53	Positive	45	162.2	125.3–199.1	0.0006
	Negative	20	77.2	43.8–110.6	
KRAS	Positive	11	94.8	33.6–156.0	0.1015
	Negative	54	144.4	111.8–177.0	
ERBB2	Positive	8	89.6	53.7–125.6	0.6204
	Negative	57	142.6	110.3–174.8	
BRAF	Positive	2	207.0	0.0–2189	0.7221
	Negative	63	133.8	105.1–162.4	
CTNNB1	Positive	1	–	–	–
	Negative	64	–	–	
PTEN	Positive	4	70.8	49.0–92.3	0.4713
	Negative	61	140.3	110.0–170.7	
CDKN2A ^b	Positive	3	199.7	62.6–336.8	0.1435
	Negative	39	133.0	103.1–162.8	
PIK3CA	Positive	5	86.0	2.78–169.2	0.3263
	Negative	60	140.2	109.6–170.9	
ALK IHC	Positive	2	38.0	0–228.6	0.0976
	Negative	63	139.2	109.8–168.5	
p53 IHC	1–3+	36	148.0	108.8–187.2	0.1429
	–	29	121.2	76.9–165.5	
Age	>68	36	112.1	85.7–138.5	0.3361
	<67	29	165.8	110.0–221.6	
Gender	Male	54	146.7	114.4–178.9	0.0296
	Female	11	83.9	21.4–146.5	
Smoking	Smoker	54	153.0	120.3–185.8	0.0022
	Never	11	52.6	38.3–67.0	
Pathology	Ad	37	115.9	75.1–156.8	0.0034
	Sq	28	162.6	122.7–202.5	
Tumor size	≥2.8	37	156.8	114.6–199.0	0.0610
	<2.7	28	108.6	71.4–145.7	
Stage	I II	54	132.9	102.6–163.3	0.9484
	III IV	11	151.4	55.6–247.1	

LN	+	15	148.5	75.7–221.4	0.8867
	–	50	132.3	100.4–164.2	
TIL	3+	17	114.5	63.0–166.1	0.2973
	1+/2+	48	143.7	108.5–178.8	
PD-L1	+	9	184.2	73.6–294.8	0.1984
	–	56	128.3	98.7–157.9	

^bCDKN2A mutation was identified using a CHP panel only, while other mutations were obtained using a CLP panel sequence. Therefore, the total number of CDKN2A analyses was 42, which differed from the CLP panel sequence analyses.

TMB: tumor-mutation burden, EGFR: EGFR gene, CLP: CLP gene, CDKN2A: cyclin-dependent kinase-inhibitor gene 2A

Table S5. Multivariate analysis for predicting TMB in EGFR-mutation-negative patients.

		β	95%CI	p value
Gender	Male	–11.071	–101.5–79.4	0.8070
Brinkman Index		0.046	–0.011–0.102	0.1130
Pathology	Sq	5.445	–57.8–68.7	0.8640
TP53	Positive	67.654	0.391–134.9	0.0490

CorrectedR²=0.105

TMB: tumor-mutation burden, EGFR: EGFR gene

Figure S1. EGFR-mutation negative (N=65)



Figure S2. Correlation between TP53 gene mutation and p53 protein expression

